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TOPICAL REVIEW

# Molecular bases of NMDA receptor subtype-dependent properties

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**Abstract** NMDA receptors (NMDARs) are a class of ionotropic glutamate receptors (iGluRs) that are essential for neuronal development, synaptic plasticity, learning and cell survival. Several features distinguish NMDARs from other iGluRs and underlie the crucial roles NMDARs play in nervous system physiology. NMDARs display slow deactivation kinetics, are highly $Ca^{2+}$ permeable, and require depolarization to relieve channel block by external $Mg^{2+}$ ions, thereby making them effective coincidence detectors. These properties and others differ among NMDAR subtypes, which are defined by the subunits that compose the receptor. NMDARs, which are heterotetrameric, commonly are composed of two GluN1 subunits and two GluN2 subunits, of which there are four types, GluN2A–D. 'Diheteromeric' NMDARs contain two identical GluN2 subunits. Gating and ligand-binding properties (e.g. deactivation kinetics) and channel properties (e.g. channel block by $Mg^{2+}$) depend strongly on the GluN2 subunit contained in diheteromeric NMDARs. Recent work shows that two distinct regions of GluN2 subunits control most diheteromeric NMDAR subtype-dependent properties: the N-terminal domain is responsible for most subtype dependence of gating and ligand-binding properties; a single residue difference between GluN2 subunits at a site termed the GluN2 S/L site is responsible for most subtype dependence of channel properties. Thus, two structurally and functionally distinct regions underlie the majority of subtype dependence of NMDAR properties. This topical review highlights recent studies of recombinant diheteromeric NMDARs that uncovered the involvement of the N-terminal domain and of the GluN2 S/L site in the subtype dependence of NMDAR properties.

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**Abbreviations** ABD, agonist-binding domain; AMPAR, AMPA receptor; CTD, C-terminal domain; iGluR, ionotropic glutamate receptor; MTS, methanethiosulfonate; NMDAR, NMDA receptor; NTD, N-terminal domain; NTD+L, NTD and NTD-ABD linker; PDB, Protein Data Bank; $P_{open}$, open probability; TMD, transmembrane domain; TMR, transmembrane region.

## Introduction

Glutamate mediates the majority of fast excitatory synaptic transmission in the central nervous system. Glutamate binds to and activates ionotropic glutamate receptors (iGluRs), which open to allow cation flux across the cell membrane. iGluRs are ligand-gated ion channels composed of four subunits organized around a central ion channel. The tertiary structure of all iGluR subunits can be described as several functionally distinct domains: an extracellular N-terminal domain (NTD; or amino-terminal domain, ATD), an extracellular agonist-binding domain (ABD; or ligand-binding domain, LBD), a transmembrane domain

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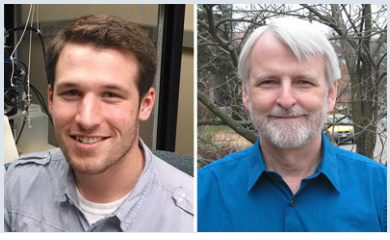

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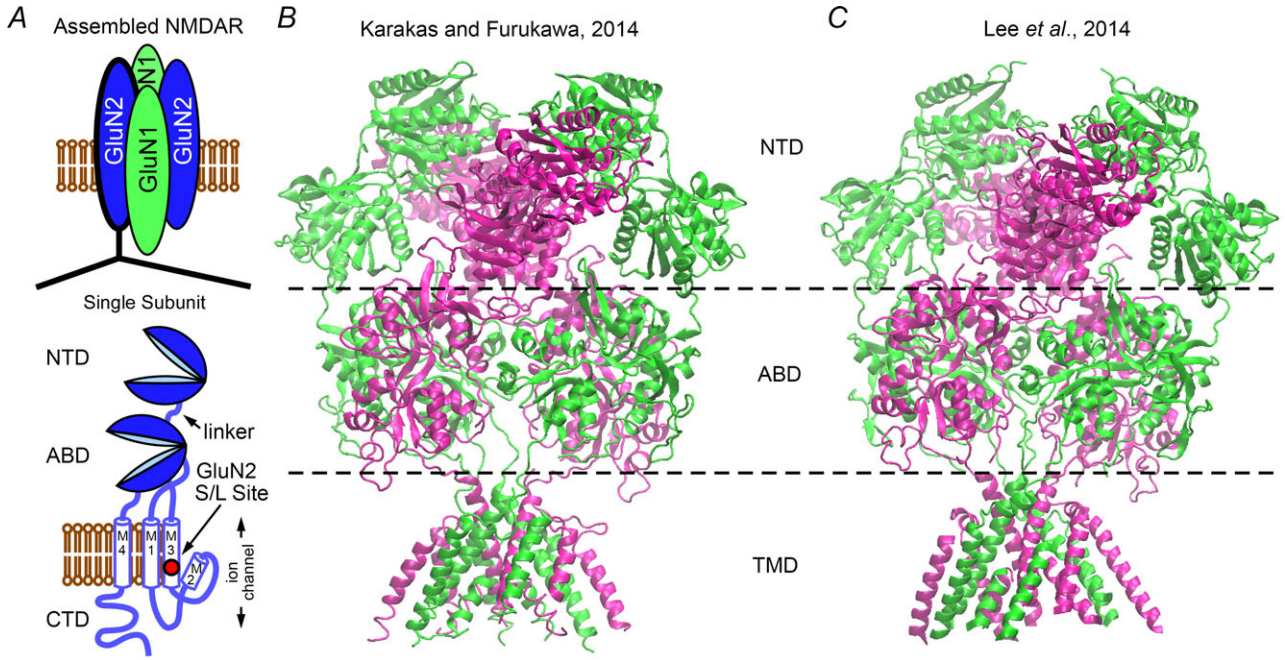
(TMD) made up of three transmembrane regions (TMRs; M1, M3 and M4) and a re-entrant pore-lining loop termed the p-loop (or M2 region) that forms the selectivity filter, and an intracellular C-terminal domain (CTD) (Fig. 1*A*) (Traynelis *et al.* 2010).

There are four classes of iGluRs: AMPA receptors (AMPARs), kainate receptors, NMDA receptors (NMDARs) and $\delta$ receptors. Receptors of each class are formed by co-assembly of homologous subunits. Subunit composition defines receptor subtypes within




*D* Diheteromeric NMDAR Subtype-Dependent Properties

| NMDAR Subtype | Gating and Ligand-Binding Properties: GluN2 NTD-Dependent | | | | | | Channel Properties: GluN2 S/L Site-Dependent | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Maximal $P_{open}$ | Agonist Potency | Deactivation Kinetics | $Zn^{2+}$ Sensitivity | Proton Sensitivity | Ifenprodil Sensitivity | $Mg^{2+}$ Sensitivity | $Ca^{2+}$ Permeability | Single-Channel Conductance | Inherent $V_m$ Dependence |
| | Low - High | Low - High | Slow - Fast | Low - High | Low - High | Low - High | Low - High | Low - High | Low - High | None - Strong |
| GluN1/2A | | | | | | | | | | |
| GluN1/2B | | | | | | | | | | |
| GluN1/2C | | | | | | | | | | |
| GluN1/2D | | | | | | | | | | |

**Figure 1. NMDAR schematic diagram, crystal structures and subtype-dependent properties**
*A*, schematic diagram of an assembled diheteromeric NMDAR (upper) with an enlarged schematic diagram of a single NMDAR subunit depicting the distinct functional domains (lower). The location of the GluN2 S/L site is indicated with a red filled circle in the M3 TMR. *B* and *C*, crystal structures of GluN1/2B NMDARs. Images in *B* (Protein Data Bank (PDB) ID: 4PE5 (Karakas & Furukawa 2014)) and in *C* (PDB ID: 4TLL (Lee *et al.* 2014)) were created using the molecular visualization program VMD (Humphrey *et al.* 1996). GluN1 subunits are shown in green and GluN2B subunits are shown in magenta. *D*, comparison of relative values of NMDAR subtype-dependent properties.

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each class of iGluR. Physiological properties, such as agonist potency, maximal channel open probability ($P_{open}$), and deactivation kinetics, can differ greatly between subtypes of each iGluR class except $\delta$ receptors, which seem not form functional receptors (Traynelis *et al.* 2010). Thus, control of the expression of specific iGluR subtypes can have enormous impact on synaptic function, membrane excitability, and activation of intracellular signalling cascades, each of which more broadly affects the physiology of neuronal circuits and systems. The tight developmental, regional and subcellular regulation of iGluR subunit expression indicates that iGluR subtypes play distinct physiological roles (Cull-Candy & Leszkiewicz, 2004).

NMDARs exhibit several properties that are unique among iGluRs, including: the requirement that both glutamate and a co-agonist, either glycine or D-serine, bind to activate the receptor (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Lerma *et al.* 1990; Schell *et al.* 1995); very slow deactivation (Forsythe & Westbrook, 1988; Lester *et al.* 1990; Partin *et al.* 1996; Swanson & Heinemann, 1998; Vicini *et al.* 1998); high permeability to $Ca^{2+}$ (MacDermott *et al.* 1986; Burnashev *et al.* 1992, 1995; Schneggenburger, 1996); and strongly voltage-dependent channel block by physiological concentrations of external $Mg^{2+}$ (Mayer *et al.* 1984; Nowak *et al.* 1984; Ascher & Nowak, 1988). Flux of $Ca^{2+}$ through NMDARs is essential for many types of synaptic plasticity, learning and memory, and cell survival (Malenka & Bear, 2004; Hardingham & Bading, 2010). Conversely, aberrant NMDAR activation is implicated in neurodegenerative diseases, schizophrenia, depression, chronic and neuropathic pain, as well as neuronal loss following ischaemia or stroke (Lau & Tymianski, 2010; Zhou & Sheng, 2013).

## Diversity of NMDAR subtypes

NMDAR subunits are encoded by seven genes. One gene encodes eight GluN1 subunit splice variants, four genes encode GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D), and two genes encode GluN3 subunits (GluN3A and GluN3B). Functional NMDARs are obligate heterotetramers thought to be assembled as a combination of two GluN1 subunits and two GluN2 and/or GluN3 subunits. Most diheteromeric NMDARs contain two GluN1 subunits and two GluN2 subunits of the same type. Triheteromeric NMDARs contain two GluN1 subunits and two GluN2 or GluN3 subunits of different identities.

The NMDAR subtype is defined by the subunits present in the receptor, which impart unique properties to each receptor subtype. This review focuses on the well-characterized diversity of the four diheteromeric NMDAR subtypes defined by the identity of the GluN2 subunits (GluN1/2A, GluN1/2B, GluN1/2C and GluN1/2D receptors; Fig. 1*D*). Many, and possibly most, native NMDARs are triheteromeric NMDAR subtypes (Luo *et al.* 1997; Al-Hallaq *et al.* 2007; Rauner & Kohr, 2010; Gray *et al.* 2011; Tovar *et al.* 2013). However, until recently, few studies have addressed triheteromeric NMDAR properties (Hatton & Paoletti, 2005; Rauner & Kohr, 2010; Tovar *et al.* 2013) due to the difficulty of studying them in isolation from other NMDAR subtypes. Very recently, exciting new approaches have been developed to study isolated triheteromeric NMDARs (Hansen *et al.* 2014; Yuan *et al.* 2014), a topic outside the scope of this review.

Heterologous expression systems, where a single NMDAR subtype can be unambiguously studied by expression of GluN1 and a single type of GluN2 subunits, have allowed extensive characterization of diheteromeric NMDAR subtype-dependent properties (Cull-Candy & Leszkiewicz, 2004; Traynelis *et al.* 2010; Paoletti *et al.* 2013). Studies in heterologous systems have revealed great diversity of diheteromeric NMDAR subtype-dependent properties including: deactivation kinetics (Monyer *et al.* 1992, 1994; Vicini *et al.* 1998), agonist potency (Kutsuwada *et al.* 1992; Priestley *et al.* 1995; Varney *et al.* 1996; Erreger *et al.* 2007; Traynelis *et al.* 2010), $Ca^{2+}$ permeability (Burnashev *et al.* 1995; Schneggenburger, 1996), voltage dependence of channel gating (Clarke, 2006; Clarke & Johnson, 2008; Clarke *et al.* 2013), sensitivity to block by external $Mg^{2+}$ (Monyer *et al.* 1994; Kuner & Schoepfer, 1996), and sensitivity to endogenous inhibitors (Traynelis *et al.* 1995; Williams, 1996; Chen *et al.* 1997; Paoletti *et al.* 1997, 2013; Traynelis *et al.* 1998). Expression and subcellular localization of NMDAR subunits varies by developmental stage, brain region and cell type (Akazawa *et al.* 1994; Monyer *et al.* 1994; Sheng *et al.* 1994). Thus, the expression of specific NMDAR subtypes can be used to tune synapses, neurons, circuits and systems through the great diversity of NMDAR subtype-dependent properties.

Despite in-depth characterization of NMDAR subtype-dependent properties (for comprehensive reviews see Cull-Candy & Leszkiewicz, 2004; Traynelis *et al.* 2010; Paoletti *et al.* 2013), for many of these properties little was known about the mechanisms that underlie their subtype dependence until recently. Here we highlight recent major advances in our understanding of the molecular bases of functional diversity among NMDAR subtype-dependent properties.

## Two categories of NMDAR subtype-dependent properties

Prior to the work described in this review, there was no clear justification for dividing the long list of NMDAR properties that depend on receptor subtype into two categories. However, recent work provides strong evidence

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that nearly all diheteromeric NMDAR subtype-dependent properties can be structurally and functionally divided into two categories: (1) gating and ligand-binding properties and (2) channel properties (Fig. 1*D*).

Two congruent studies in 2009 revealed that the GluN2 NTD controls the NMDAR subtype dependence of gating and ligand-binding properties (Gielen *et al.* 2009; Yuan *et al.* 2009). These two studies identified the following NTD-dependent properties: maximal $P_{open}$, agonist potency, deactivation kinetics, and sensitivity to the endogenous inhibitors $Zn^{2+}$ and protons. Other studies identified inhibition by ifenprodil (Fig. 1*D*) (Perin-Dureau *et al.* 2002; Malherbe *et al.* 2003; Ng *et al.* 2008; Karakas *et al.* 2009) and voltage- and glycine-independent potentiation by polyamines (Gallagher *et al.* 1997; Masuko *et al.* 1999; Han *et al.* 2008; Mony *et al.* 2011) as additional NTD-dependent ligand-binding properties that differ among NMDAR subtypes. In contrast, the residue at a single site near the intracellular end of the M3 region of GluN2 subunits, a serine (S) in GluN2A (S632; residue numbering used here begins at the start methionine) or GluN2B (S633) and a leucine (L) in GluN2C (L643) or GluN2D (L657) (the GluN2 S/L site; Fig. 1*A*), controls diheteromeric NMDAR subtype-dependent channel properties (Siegler Retchless *et al.* 2012; Clarke *et al.* 2013). The GluN2 S/L site was shown to control the subtype dependence of the following properties: $Mg^{2+}$ sensitivity, $Ca^{2+}$ permeability, single-channel conductance, and inherent voltage dependence of channel gating (Fig. 1*D*). Unlike gating and ligand-binding properties, channel properties of GluN1/2A and GluN1/2B receptors are very similar, channel properties of GluN1/2C and GluN1/2D receptors are very similar, but channel properties of GluN1/2A and GluN1/2B receptors differ strongly from those of GluN1/2C and GluN1/2D receptors (Fig. 1*D*). The residue at the GluN2 S/L site determines whether the channel properties of an NMDAR resemble properties of GluN1/2A and GluN1/2B receptors (GluN1/2A-like) or resemble properties of GluN1/2C and GluN1/2D receptors (GluN1/2D-like) (Siegler Retchless *et al.* 2012; Clarke *et al.* 2013).

## The GluN2 NTD controls gating and ligand-binding properties

Diheteromeric NMDARs display wide functional variation in gating and ligand-binding properties. For instance, GluN1/2A receptors have a maximal $P_{open}$ of ∼0.5, GluN1/2B receptors have a maximal $P_{open}$ of ∼0.1, and GluN1/2C and GluN1/2D receptors have a maximal $P_{open}$ of ∼0.01 (Wyllie *et al.* 1998; Chen *et al.* 1999; Erreger *et al.* 2005; Dravid *et al.* 2008). Gielen *et al.* (2009) and Yuan *et al.* (2009) demonstrated that the NMDAR subtype dependence of maximal $P_{open}$ and other gating and ligand-binding properties is largely due to variation at the GluN2 NTD and the linker region that connects the GluN2 NTD to the ABD (NTD-ABD linker). Both studies took advantage of mutant receptors containing GluN2 subunits with either the NTD deleted (ΔNTD) or the NTD and NTD-ABD linker deleted (ΔNTD+L), as well as chimeric receptors containing GluN2 subunits in which the GluN2 NTD, or the NTD and NTD-ABD linker (NTD+L), was replaced with the NTD or NTD+L of another GluN2 subunit (Fig. 2*A*).

Gielen *et al.* (2009) showed that the dependence of maximal $P_{open}$ on NMDAR subtype was abolished in GluN2(ΔNTD) subunit-containing receptors. Examination of GluN1/2B(2A-NTD) and GluN1/2A(2B-NTD) receptors revealed that exchanging NTDs does not result in an exchange of maximal $P_{open}$ (Gielen *et al.* 2009). However, examination of GluN1/2B(2A-NTD+L) and GluN1/2A(2B-NTD+L) receptors revealed that exchanging the NTD+L does result in an exchange of maximal $P_{open}$ (Fig. 2*B*).

Yuan *et al.* (2009) examined the influence of the GluN2 NTD+L on steady-state $P_{open}$ in saturating agonist concentrations using mutant GluN2 subunits in which the NTD+L were removed, as opposed to just the NTD. A distinction of this study from Gielen *et al.* (2009) is the investigation of differences between GluN1/2A and GluN1/2D receptors, which exhibit much greater differences in gating and ligand-binding properties than GluN1/2A and GluN1/2B receptors. GluN1/2A(2D-NTD+L) receptors displayed $P_{open}$ values in saturating agonist concentrations far lower than wild-type GluN1/2A receptors and similar to wild-type GluN1/2D receptors, whereas GluN1/2D(2A-NTD+L) receptors displayed $P_{open}$ values nearly 5-fold greater than wild-type GluN1/2D receptors, although still far below wild-type GluN1/2A receptor values (Yuan *et al.* 2009). Interestingly, there were intermediate effects on $P_{open}$ when only the NTD-ABD linker was interchanged between GluN2A and GluN2D subunits. These studies argue strongly for a fundamental role of the NTD and the NTD-ABD linker in determining maximal $P_{open}$. Importantly, Yuan *et al.* (2009) demonstrated that the presence or identity of the NTD+L did not affect single-channel conductance.

The identity of the GluN2 NTD+L also affects agonist potency and deactivation kinetics (Yuan *et al.* 2009). The glutamate potency of all receptors that contained a GluN2(ΔNTD+L) subunit were indistinguishable from wild-type receptors (Fig. 2*C*). The glycine potency of GluN1/2A and GluN1/2A(ΔNTD+L) did not differ, whereas the glycine potency of GluN1/2B(ΔNTD+L), GluN1/2C(ΔNTD+L), and GluN1/2D(ΔNTD+L) receptors was lower than for corresponding wild-type receptors. The time constant of deactivation following rapid removal of glutamate ($\tau$)

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also differed between wild-type receptors and receptors containing GluN2(ΔNTD+L) subunits. Importantly, for glutamate $EC_{50}$, glycine $EC_{50}$ and $\tau$ values, chimeric GluN1/2A(2D-NTD+L) receptors resembled GluN1/2D more closely than GluN1/2A receptors, and chimeric GluN1/2D(2A-NTD+L) receptors resembled GluN1/2A more closely than GluN1/2D receptors (Fig. 2*C* and *D*) (Yuan *et al.* 2009).

Gielen *et al.* (2009) also investigated NMDAR subtype dependence of sensitivity to the endogenous allosteric inhibitors $Zn^{2+}$ and protons. $Zn^{2+}$ inhibits GluN1/2A receptors with high affinity (in the nanomolar range) and inhibits GluN1/2B receptors in the micromolar range (Fig. 1*D*) (Williams, 1996; Chen *et al.* 1997; Paoletti *et al.* 1997; Traynelis *et al.* 1998; Rachline *et al.* 2005). A $Zn^{2+}$ binding site is in the bilobed cleft of both the GluN2A and GluN2B NTDs, where binding of $Zn^{2+}$ stabilizes a closed cleft conformation of the NTD (Choi & Lipton, 1999; Fayyazuddin *et al.* 2000; Low *et al.* 2000; Paoletti *et al.* 2000; Choi *et al.* 2001; Rachline *et al.* 2005; Karakas *et al.* 2009; Stroebel *et al.* 2011). Crystallography of $Zn^{2+}$ bound to the GluN2B NTD showed that $Zn^{2+}$ directly contacts a histidine and a glutamate residue in the GluN2B NTD (Karakas *et al.* 2009). The two homologous residues in the GluN2A NTD (a histidine and an asparagine residue) are thought to coordinate $Zn^{2+}$, along with at least one additional histidine residue, and possibly a lysine and a glutamate residue (Choi & Lipton,

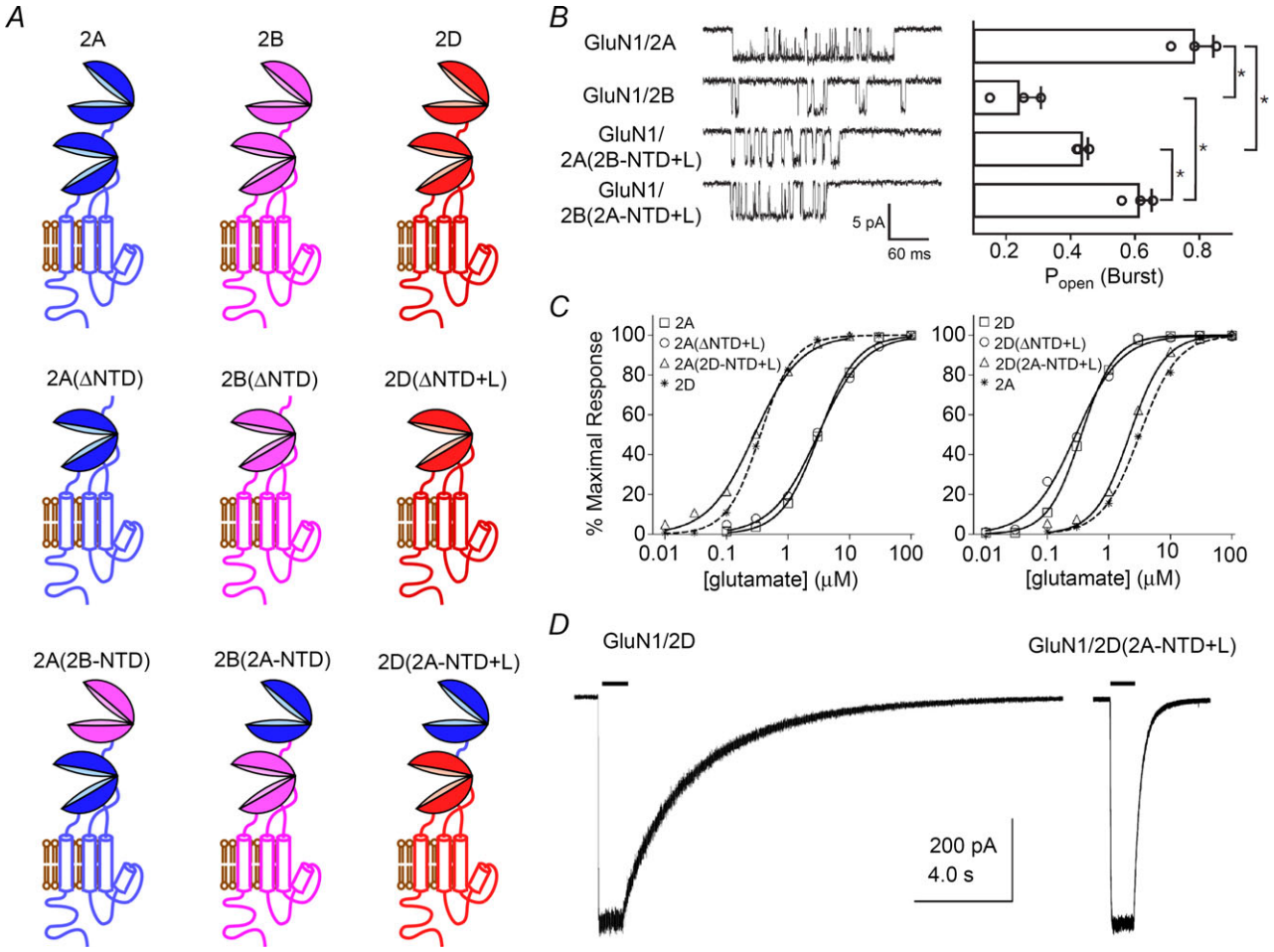


**Figure 2. The GluN2 NTD and adjacent linker region control gating and ligand-binding properties**
*A*, schematic diagrams of wild-type GluN2 subunits (upper), examples of GluN2 subunits with deletions (middle), and examples of chimeric GluN2 subunits with domains interchanged between GluN2 subunits (lower). Colours of domains correspond to the GluN2 subunits (upper). *B*, single-channel records of wild-type or chimeric GluN2 subunit-containing receptors (left) and the $P_{open}$ during bursts of channel openings, which was used as an estimate of maximal $P_{open}$ (right). Panel was modified from Gielen *et al.* (2009). *C*, glutamate concentration–response relations of wild-type, GluN2(ΔNTD+L), and chimeric GluN2(NTD+L) subunit-containing receptors. Panel was modified from Yuan *et al.* (2009), with permission. *D*, whole-cell recording of wild-type and chimeric GluN1/2D(2A-NTD+L) receptors showing the time course of deactivation following 1 s applications of 1 mM glutamate (black bars). Panel was modified from Yuan *et al.* (2009), with permission.

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1999; Fayyazuddin *et al.* 2000; Low *et al.* 2000). The additional GluN2A NTD residues interacting with $Zn^{2+}$ are thought to be responsible for the higher affinity of GluN1/2A receptors for $Zn^{2+}$. In contrast to the $Zn^{2+}$ binding site, the location of the proton sensor is not known. Several mutations in the NTD, ABD and pore regions have been shown to affect proton sensitivity (Low *et al.* 2003; Gielen *et al.* 2008); however, interpretation of mutant studies is complicated because high affinity $Zn^{2+}$ inhibition enhances proton sensitivity (Low *et al.* 2000; Erreger & Traynelis, 2008). Nevertheless, protons are thought to mediate their inhibitory effect through associations with regions near the channel gate (Low *et al.* 2003; Traynelis *et al.* 2010).

Chimeric GluN1/2D(2A-NTD+L) and GluN1/2B(2A-NTD+L) receptors exhibited $Zn^{2+}$ sensitivity nearly identical to wild-type GluN1/2A receptors (Gielen *et al.* 2009). Surprisingly, GluN1/2B(2A-NTD) receptors were significantly more sensitive to $Zn^{2+}$ than GluN1/2A receptors, suggesting that the GluN2B NTD-ABD linker facilitates NTD cleft closure. Sensitivity to protons was unexpectedly affected by the identity of the GluN2 NTD. GluN1/2A(ΔNTD) and GluN1/2B(ΔNTD) receptors did not display NMDAR subtype dependence of proton sensitivity as displayed in wild-type GluN1/2A and GluN1/2B receptors. Furthermore, examination of GluN1/2A(2B-NTD+L) and GluN1/2B(2A-NTD+L) receptors revealed that exchanging the NTD+L results in exchanged proton sensitivity. These data support the role of the GluN2 NTD and NTD-ABD linker in mediating the effect of $Zn^{2+}$ on channel gating (Erreger & Traynelis, 2008) and the accessibility of protons to the proton sensor (Gielen *et al.* 2009).

The GluN2 NTD also confers sensitivity to the synthetic allosteric modulator ifenprodil and its derivatives, such as Ro 25-6981, which display >100-fold selectivity for GluN1/2B receptors over other diheteromeric NMDAR subtypes (Williams, 1993; Traynelis *et al.* 2010). Like high affinity $Zn^{2+}$ binding to the GluN2A NTD, ifenprodil sensitivity is conferred by the GluN2B NTD (Perin-Dureau *et al.* 2002; Malherbe *et al.* 2003; Ng *et al.* 2008; Karakas *et al.* 2011). However, unlike $Zn^{2+}$, which binds in the cleft of the NTD (Karakas *et al.* 2009), a recent crystal structure showed that ifenprodil binds to the interface between the GluN1 and GluN2B NTDs (Karakas *et al.* 2011). Interestingly, only a single residue (an isoleucine (I) in GluN2B and a methionine (M) in GluN2A) differs between the GluN2B NTD (I111) and the GluN2A NTD (M112) in the ifenprodil binding pocket. Receptors containing mutated GluN2B(I111M) or GluN2A(M112I) subunits do not exhibit abolished or augmented ifenprodil sensitivity compared to the mutant receptors, respectively (Karakas *et al.* 2011). Therefore, the mechanism of ifenprodil selectivity of GluN1/2B receptors over GluN1/2A receptors is still not fully understood.

To investigate the mechanism by which ligands that bind to the NTD influence NMDAR gating and ligand-binding properties, Gielen *et al.* (2009) introduced cysteine residues deep into the NTD cleft, creating GluN2A(Y281C) and GluN2B(Y282C) subunits. Cysteine modification by methanethiosulfonate (MTS) reagents of varying size was then used to lock open the NTD cleft. Potentiation of GluN1/2B(Y282C) receptor currents by MTS reagent modification increased with increasing MTS reagent size. A similar, but lesser increase in potentiation of GluN1/2A(Y281C) receptor currents by MTS reagent modification was seen with increasing MTS reagent size. Based on these data, Gielen *et al.* (2009) proposed a model in which oscillation of GluN2 NTDs between open and closed conformations in the absence of NTD ligands determines the maximal $P_{open}$ of a receptor. GluN1/2A receptors were hypothesized to exhibit a higher maximal $P_{open}$ than GluN1/2B receptors because of higher occupancy by the GluN2A NTD of the open cleft conformation. An alternative possibility is that the GluN2A NTD might adopt a more open conformation than the GluN2B NTD when no ligand is bound, and MTS reagent modification may lead to greater than normal NTD opening. In contrast to the NTD, when the bilobed ABD cleft of GluN2A subunits (Furukawa *et al.* 2005) is locked closed using disulfide bridges, maximal $P_{open}$ of GluN1/2A receptors increases (Kussius & Popescu, 2010).

The gating and ligand-binding properties of receptors containing chimeric GluN2(NTD+L) subunits created by Gielen *et al.* (2009) and Yuan *et al.* (2009) were not fully converted to the properties of subtypes containing the GluN2 subunits that contributed the NTD+L. In addition, results with truncated receptors were unpredictable; truncation had no significant effect on some receptor properties, while strongly modifying other receptor properties. These and other data indicate that regions other than the NTD+L contribute significantly to the NMDAR subtype dependence of gating and ligand-binding properties. Another NMDAR domain that appears likely to contribute is the ABD (Erreger *et al.* 2007; Chen *et al.* 2008), whereas the CTD appears unlikely to contribute (Maki *et al.* 2012; Martel *et al.* 2012; Punnakkal *et al.* 2012; Ryan *et al.* 2013). Nevertheless, Gielen *et al.* (2009) and Yuan *et al.* (2009) demonstrate the critical importance of the GluN2 NTD and NTD-ABD linker in controlling diheteromeric NMDAR subtype dependence of gating and ligand-binding properties.

During final revisions of this review, two separate crystal structures of intact diheteromeric GluN1/2B receptors were published within weeks of one another (Fig. 1*B* and *C*) (Karakas & Furukawa, 2014; Lee *et al.* 2014). Both structures revealed NMDAR subunit arrangement and organization similar to the homomeric GluA2 AMPAR crystal structure (Sobolevsky *et al.* 2009). However, the shape of the GluN1/2B NMDAR crystal structures differed

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from the GluA2 AMPAR crystal structure, with, for example, the NTD and ABD much more closely associated in the GluN1/2B NMDAR crystal structures (Sobolevsky *et al.* 2009; Karakas & Furukawa, 2014; Lee *et al.* 2014). The close association of the NTD and ABD in the GluN1/2B NMDAR crystal structures suggests that ligand binding to the NTD may affect the ABD through multiple contacts in addition to the NTD-ABD linker (Karakas & Furukawa, 2014; Lee *et al.* 2014). Assessment of NTD-ABD interactions in the GluN1/2B NMDAR crystal structures may be complicated by modifications designed to increase NMDAR stability. Of note, deletions were made within the GluN2B NTD-ABD linker, and cysteine residues introduced in GluN2B NTDs formed intersubunit cross-links that nearly eliminated receptor activity (Karakas & Furukawa, 2014; Lee *et al.* 2014). However, the GluN1/2B NMDAR crystal structures represent a fundamental advance that will be invaluable in determining how the NTD influences NMDAR gating and ligand-binding properties.

## The GluN2 S/L site controls channel properties

The identity of the GluN2 NTD+L does not affect single-channel conductance (Yuan *et al.* 2009), and whether the identity of the GluN2 NTD or NTD+L affects other channel properties was not investigated. Therefore, NMDAR subtype dependence of channel properties may be controlled by other regions of the receptor.

Previous studies that mostly utilized chimeric subunits implicated the TMD and the ABD in controlling the NMDAR subtype dependence of channel properties (Kuner & Schoepfer, 1996; Wrighton *et al.* 2008; O'Leary & Wyllie, 2009). Kuner & Schoepfer (1996) investigated the role of the entire TMD, subregions of the TMD, and part of the ABD in the NMDAR subtype dependence of $Mg^{2+}$ sensitivity. They found that parts of the M1, M2, M3 and M4 regions in GluN2 subunits all contribute to the subtype dependence of $Mg^{2+}$ sensitivity. Wrighton *et al.* (2008) found that the M1–M3 regions, and to a lesser extent the ABD, were responsible for determining subtype dependence of $Mg^{2+}$ sensitivity. O'Leary & Wyllie (2009) showed that the M1–M3 regions determine subtype dependence of single-channel conductance in addition to $Mg^{2+}$ sensitivity. Therefore, multiple structural elements were found to contribute to NMDAR subtype dependence of channel properties.

Siegler Retchless *et al.* (2012) investigated the structural determinants of GluN1/2A-like and GluN1/2D-like channel properties by mutating single residues. Mutations were introduced at sites within the TMD where GluN2A and GluN2B subunits express the same residue, GluN2C and GluN2D subunits express the same residue, but the GluN2A and GluN2B subunit residue differs from the GluN2C and GluN2D subunit residue.

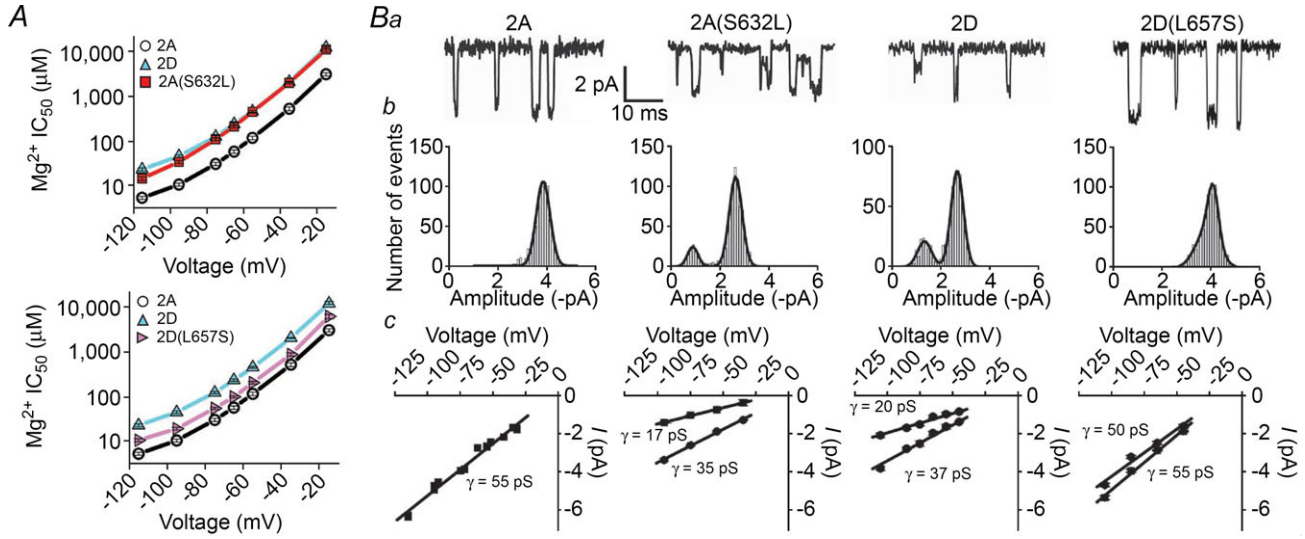


**Figure 3. The GluN2 S/L site controls channel properties**
*A*, voltage dependence of $Mg^{2+}$ $IC_{50}$ values of wild-type receptors and receptors with a mutation at the GluN2 S/L site (see Fig. 1*A*). $Mg^{2+}$ $IC_{50}$ values of GluN1/2A(S632L) receptors resembled those of GluN1/2D receptors, whereas $Mg^{2+}$ $IC_{50}$ values of GluN1/2D(L657S) receptors resembled those of GluN1/2A receptors. *B*, single-channel records (*a*), current amplitude histograms (*b*), and current–voltage relations (*c*) of wild-type receptors and receptors with a mutation at the GluN2 S/L site. Slopes of single-channel current–voltage relations were used to determine single-channel conductance ($\gamma$) in *c*. Single-channel current amplitude histograms and $\gamma$ values of GluN1/2A(S632L) receptors resembled those of GluN1/2D receptors, whereas single-channel current amplitude histograms and $\gamma$ values of GluN1/2D(L657S) receptors resembled those of GluN1/2A receptors. Panels were taken from Siegler Retchless *et al.* (2012).

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Mutations of residues at a single site, the GluN2 S/L site, were found to control NMDAR subtype dependence of channel properties. GluN1/2A(S632L) receptors, in which the residue at the GluN2 S/L site is changed from the GluN2A S to the GluN2D L, exhibit NMDAR channel properties that are GluN1/2D-like (Fig. 3*A* and *B*). Conversely, GluN1/2D(L657S) receptors, in which the GluN2D L at the GluN2 S/L site is changed to the GluN2A S, exhibit NMDAR channel properties that are GluN1/2A-like (Fig. 3*A* and *B*) (Siegler Retchless *et al.* 2012; Clarke *et al.* 2013). The single residue at the GluN2 S/L site was found to control NMDAR subtype dependence of: $Mg^{2+}$ sensitivity (Fig. 3*A*); $Ca^{2+}$ permeability; single-channel conductance, including the conductance of subconductance states (Fig. 3*B*); and inherent voltage-dependent gating of NMDARs (Siegler Retchless *et al.* 2012; Clarke *et al.* 2013). Kinetic analysis of single-channel recordings from GluN1/2A(S632L) receptors did not reveal significant differences from GluN1/2A receptors, consistent with the conclusion that the GluN2 NTD controls NMDAR subtype dependence of gating kinetics.

Although the GluN2 S/L site powerfully affects NMDAR subtype-dependent channel properties, interconversion of channel properties between GluN1/2D-like and GluN1/2A-like by GluN2 S/L site substitutions was incomplete. Thus, regions in addition to the GluN2 S/L site influence NMDAR subtype dependence of channel properties. As noted above, other parts of the TMD and the ABD were found to contribute to NMDAR subtype dependence of $Mg^{2+}$ sensitivity (Kuner & Schoepfer, 1996; Wrighton *et al.* 2008; O'Leary & Wyllie, 2009). Taken together, studies that examined the NMDAR subtype dependence of channel properties suggest that the GluN2 S/L site is the major determinant of subtype dependence, with other parts of the TMD, and the ABD, playing smaller roles.

Because the GluN2 S/L site is located at the base of the M3 TMR, it is unlikely to interact directly with ions in the pore. Therefore, the GluN2 S/L site probably affects NMDAR subtype-dependent channel properties through interactions with residues that are closer to the pore.

## The GluN2 S/L site controls channel properties through subunit–subunit interactions

Until recently, neither an intact NMDAR crystal structure nor a crystal structure of the NMDAR TMD had been published. Therefore, to aid in understanding the mechanism by which the GluN2 S/L site controls NMDAR subtype-dependent channel properties, Siegler Retchless *et al.* (2012) created homology models of the GluN1/2A M2 p-loop and M3 TMR (GluN1/2A M2–M3; Fig. 4). Homology models take advantage of structural homology of previously crystallized proteins with a protein of interest that has not yet been crystallized. Homology models allow prediction of the structure of the protein of interest based on sequence alignment and hypothesized structural homologies.

In 2009, the first nearly complete iGluR crystal structure was published (Sobolevsky *et al.* 2009). The GluA2 AMPAR crystal structure led to many breakthroughs concerning the function and structural organization of iGluRs, including NMDARs (e.g. Traynelis *et al.* 2010; Salussolia *et al.* 2011; Riou *et al.* 2012). However, despite the utility of the GluA2 AMPAR crystal structure for answering structural and functional questions, the extended region of the M2 p-loop was not resolved (Sobolevsky *et al.* 2009). Siegler Retchless *et al.* (2012) therefore utilized crystal structures of more distantly related ion channels as a basis for a GluN1/2A M2–M3 homology model. Several earlier studies had based NMDAR channel homology models on the crystal structure of the bacterial potassium channel KcsA (Doyle *et al.* 1998), and predictions of the homology models had been validated with physiological experiments (Wood *et al.* 1995; Panchenko *et al.* 2001; Tikhonov, 2007). Another crystallized channel, the cyclic nucleotide-gated channel NaK, shares great sequence homology and structural similarity with potassium channels (Shi *et al.* 2006), but, like NMDARs, is permeable to $Na^{+}$, $K^{+}$ and $Ca^{2+}$ (Shi *et al.* 2006; Alam *et al.* 2007). Siegler Retchless *et al.* (2012) chose to base their GluN1/2A M2–M3 homology model on the NaK channel structure. Karakas & Furukawa (2014) and Lee *et al.* (2014) found that homologous portions of the TMDs of their GluN1/2B NMDAR crystal structures displayed high structural similarity to corresponding regions of the KcsA channel, further supporting the use of potassium and related channel structures for NMDAR homology modelling.

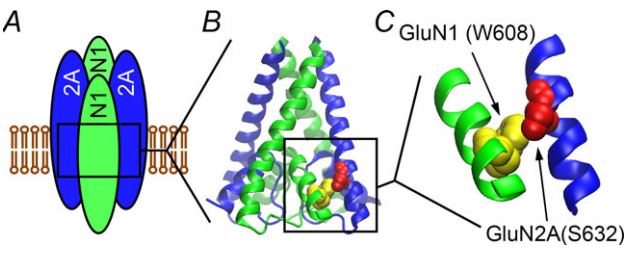


**Figure 4. NaK channel-based GluN1/2A M2–M3 homology model**
*A*, schematic diagram of an assembled NMDAR. *B*, all four subunits of the NaK-based GluN1/2A M2–M3 homology model (Siegler Retchless *et al.*, 2012) magnified from the NMDAR schematic diagram in *A*. Regions of GluN1 are shown in green, and regions of GluN2A are shown in blue. One of the two GluN1(W608) residues is shown as space-filling model in yellow, and the adjacent GluN2A(S632) residue is shown as space-filling model in red. *C*, enlarged view of GluN2 S/L site interaction with W608 in the GluN1 M2 region.

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The NaK channel-based GluN1/2A M2–M3 homology model was developed with a GluN1-GluN2A-GluN1-GluN2A arrangement around the pore based on Sobolevsky *et al.* (2009), an arrangement that subsequently has been further supported (Rambhadran *et al.* 2010; Salussolia *et al.* 2011; Riou *et al.* 2012; Karakas & Furukawa, 2014; Lee *et al.* 2014). The NaK channel-based GluN1/2A M2–M3 model predicted that GluN2A(S632) is very close to two tryptophan residues in the adjacent GluN1 subunit: GluN1(W608) and GluN1(W611), which are in the $\alpha$-helical portion of the GluN1 M2 p-loop (Fig. 4*B* and *C*) (Siegler Retchless *et al.* 2012). Using mutant cycle analyses (Hidalgo & MacKinnon, 1995; Schreiber & Fersht, 1995), Siegler Retchless *et al.* (2012) demonstrated coupling between GluN2A(S632) and GluN1(W608), but not between GluN2A(S632) and GluN1(W611). Thus, the authors concluded that the identity of the residue at the GluN2 S/L site is likely to control NMDAR subtype-dependent channel properties through a subunit–subunit interaction between the GluN2 M3 and GluN1 M2 $\alpha$-helices (Fig. 4*C*) (Siegler Retchless *et al.* 2012).

Siegler Retchless *et al.* (2012) also developed a GluN1/2A M2–M3 homology model based on the GluA2 AMPAR crystal structure. Importantly, the AMPAR-based GluN1/2A M2–M3 homology model did not predict close proximity of the GluN2 S/L site to GluN1(W608) (Siegler Retchless *et al.* 2012). In the NaK channel-based GluN1/2A M2–M3 homology model, the side chains of GluN1(W608) and GluN2A(S632) have a minimum separation of 3.5 Å (Fig. 4*C*). In the AMPAR-based GluN1/2A M2–M3 homology model, the side chains of GluN1(W608) and GluN2A(S632) have a minimum separation of 12.2 Å. Although mutant cycle analysis provides only an indirect gauge of proximity of residues, coupling is likely to occur only for residues with side chains separated by less than 7 Å (Schreiber & Fersht, 1995). Thus, the mutant cycle data are more consistent with the NaK channel-based than the AMPAR-based model. Possible explanations for why the distantly related NaK channel may better model the GluN1/2A M2–M3 regions than the more closely related AMPAR include: (1) the structure of the M2–M3 regions of NMDARs simply may resemble more closely membrane regions of potassium channels and closely-related channels than AMPARs; (2) the limited resolution of the M2 region of homomeric GluA2 AMPAR crystal structure may have led to inaccurate placement of the M2 $\alpha$-helix.

The pore-lining regions in the recently published GluN1/2B NMDAR crystal structures, like the GluA2 AMPAR crystal structure, were not well resolved. However, Lee *et al.* (2014) were able to position residues in the majority of the TMD, including the M2 p-loops, in their structure 2 (PDB ID: 4TLM). We measured the minimum separation between GluN1(W608) and the GluN2B residue homologous to GluN2A(S632) in structure 2. The result, 7.5 Å, is between the minimum separation in the NaK channel-based (3.5 Å) and the AMPAR-based (12.2 Å) GluN1/2A M2–M3 homology models. As Lee *et al.* (2014) were careful to point out, atom positioning in the pore region was not precise; measurements of distances between residues near the pore therefore are subject to substantial uncertainty. The limited resolution of the pore regions of currently available iGluR crystal structures suggest that high-resolution NaK and related channel crystal structures remain valuable resources for modelling the TMD of iGluRs.

## Conclusion

The functional properties of diheteromeric NMDAR subtypes depend on the identity of the GluN2 subunits present in the receptor. Recent studies provide strong evidence for grouping diheteromeric NMDAR subtype-dependent properties into two categories based on distinct structural determinants and functional characteristics. Gating and ligand-binding properties are primarily controlled by the identity of the GluN2 NTD and NTD-ABD linker (Gallagher *et al.* 1997; Perin-Dureau *et al.* 2002; Gielen *et al.* 2009; Yuan *et al.* 2009; Mony *et al.* 2011), whereas channel properties are primarily controlled by the identity of the residue at the GluN2 S/L site (Siegler Retchless *et al.* 2012; Clarke *et al.* 2013). Previous work has suggested that subunit–subunit interactions have profound effects on gating and ligand-binding properties (Monyer *et al.* 1994; Vicini *et al.* 1998; Regalado *et al.* 2001; Schorge *et al.* 2005; Vance *et al.* 2012). Similarly, the influence of the GluN2 S/L site on channel properties depends on a subunit–subunit interaction between the GluN2 M3 TMR and the GluN1 M2 p-loop. Thus, NMDAR subunit–subunit interactions are critically important in determining diheteromeric NMDAR subtype-dependent properties. Use of receptor crystal structures (e.g. Karakas & Furukawa, 2014; Lee *et al.* 2014) and structural models will be essential to our understanding of the interdomain and intersubunit interactions that play fundamental roles in NMDAR subtype-dependent properties, and most other aspects of iGluR function.

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### Competing interests

The authors have no competing interests.

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